Inositol phosphoceramide synthase is a regulator of intracellular levels of diacylglycerol and ceramide during the G_1 to S transition in *Saccharomyces cerevisiae*

Jorge CERBÓN[1], Alejandro FALCON, Carlos HERNÁNDEZ-LUNA and David SEGURA-COBOS
Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del IPN, 07000 México, D.F., México

We recently reported that DAG (diacylglycerol) generated during sphingomyelin synthesis plays an important role in protein kinase C activation and cell proliferation in Madin–Darby canine kidney cells [Cerbon and Lopez-Sanchez (2003) Biochem. J. **373**, 917–924]. In yeast cells, IPC (inositol phosphoceramide) synthase catalyses the transfer of phosphoinositol from phosphatidylinositol to ceramide to form IPC and generates DAG. In the present study, we found that, during the G_1 to S transition after N_2-starvation, there was a significant increase in the synthesis of IPC accompanied by a progressive increase (up to 6-fold) in the level of DAG. The increased DAG levels coincided with decrements in ceramide and sphingoid base levels, conditions that are adequate for the activation of putative protein kinase C required for the G_1 to S transition and proliferation of yeast cells. To separate the role of DAG generated during IPC synthesis from that originating from other sources, we utilized β-chloroalanine and myriocin, inhibitors of serine:palmitoyl-CoA transferase, the first committed step in sphingolipid synthesis, to avoid accumulation of sphingolipid intermediates. When the synthesis of sphingolipids was inhibited, DAG accumulation was significantly decreased and the G_1 to S transition was blocked; such blockage was avoided by metabolic complementation with phytosphingosine. The DAG/ceramide ratio was 0.27 and it changed to 2.0 during growth re-initiation, suggesting that the synthesis of phosphosphingolipids could act to switch growth arrest (increased ceramide) to a mitogenic signal (increased DAG), and that this signalling process is preserved in yeast and mammalian cells.

Key words: ceramide, diacylglycerol (DAG) generation, G_1 to S transition, inositol phosphoceramide (IPC) synthesis, nitrogen starvation, sphingolipid.

INTRODUCTION

Lipids play a central role in signal transduction and in yeast cells, among the signalling lipid molecules, ceramide and DAG (diacylglycerol) have been found to play a role in the regulation of cell growth and viability. A ceramide-activated protein phosphatase mediates ceramide-induced G_1 arrest in *Saccharomyces cerevisiae* [1], and the addition of C_2 ceramide to these yeasts resulted in growth arrest [2]. Similarly, ceramide accumulation is accompanied by cell death in IPC (inositol phosphoceramide) synthesis-deficient yeast mutants [3]. Elevation of DAG levels has been observed before or at the start of transition, the presumptive step of PKC (protein kinase C) activation in response to Cdc28 [4]. The best-studied pathway of DAG generation is through phosphoinositide breakdown with concomitant increases in DAG and intracellular Ca^{2+} [secondary to IP_3 (inositol trisphosphate) generation], and activation of PKC [5]. The addition of ammonium sulphate to starved yeast (G_1) cells results in a rapid 3–4-fold increase in IP_3 and DAG; however, DAG levels exceeded those of extractable IP_3 by 10–15-fold, indicating activation of other phospholipases [6]. In addition, when inositol lipid metabolism was studied in a series of Cdc (cell division cycle) mutants of *S. cerevisiae*, no evidence for the formation of inositol polyphosphates was found [7]. Other mechanisms of DAG production are mediated by the consecutive action of PC (phosphatidylcholine)-specific PLD (phospholipase D) and phosphatidate phosphohydrolase and by PC hydrolysis through a PC-specific phospholipase C. In *S. cerevisiae*, homozygous diploid disruption mutants of PLD/SPO 14 have no apparent growth phenotype. They are viable and grow but they are incapable of sporulation, and a second PLD activity has been detected [8], which in contrast to pld1p/spo14p, is Ca^{2+}-dependent but independent of phosphatidylinositol 4,5-bisphosphate. The genes encoding this second PLD have not yet been identified. Alternatively, phosphatidate phosphatase (Lpp1p) and DAG pyrophosphatase (Dpp1p) appear to be involved in DAG formation. However, strains with deletions in DPP1, LPP1 and the dpp1/lpp1 double mutant are viable and do not exhibit any obvious growth defects [9]. Also, mutants have been isolated which synthesized virtually no PC [10], suggesting that the DAG necessary for signalling may arise from other sources.

In contrast, yeast mutants incapable of synthesizing sphingolipids (due to impaired synthesis of sphingoid bases), show inability to grow and lose viability if not supplemented, suggesting that there are vital functions for IPCs in yeast [11]. IPC synthase transfers inositol phosphate from PI (phosphatidylinositol) to ceramide to form IPC [3] and to generate DAG. IPC synthase therefore has the ability to directly regulate, in opposite directions, ceramide and DAG levels, potentially controlling opposite cellular processes such as cell proliferation and growth arrest. In this study, we investigated the synthesis of IPCs during growth re-initiation in *S. cerevisiae* G_0/G_1 (stationary phase) cells and in G_1

Abbreviations used: Aur-A, aureobasidin A; CSM, complete synthetic medium; DAG, diacylglycerol; DHS, D-erythrosphingosine; IP_3, inositol trisphosphate; IPC, inositol phosphoceramide; $M(IP)_2C$, mannose (inositol phosphate)$_2$ ceramide; MIPC, mannose inositol phosphoceramide; PC, phosphatidylcholine; PHS, phytosphingosine; PI, phosphatidylinositol; PKC, protein kinase C; PLD, phospholipase D; PS, phosphatidylserine; SPT, serine:palmitoyl-CoA transferase.

[1] To whom correspondence should be addressed (email jcerbon@mail.cinvestav.mx).

(N_2-starved) cells. We analysed the consequences of inactivation of SPT (serine:palmitoyl-CoA transferase) and IPC synthase in *S. cerevisiae*, which are considered to be rate-limiting enzymes in the sphingolipid biosynthesis pathway [12]. β-Chloroalanine and myriocin [13], which are inhibitors of SPT (EC 2.3.1.50), the first committed step of sphingolipid synthesis, were used. Blocking the first step of this pathway would result in progressive inhibition of the incorporation of labelled precursors [^{3}H]palmitate, [^{32}P]P_i and [^{3}H]inositol into IPC, MIPC (mannose IPC) and M(IP)$_2$C [mannose (inositol phosphate)$_2$ ceramide].

Furthermore, since *S. cerevisiae* utilizes exogenous long-chain bases in the synthesis of sphingolipids [11], supplementation of the growth medium with PHS (phytosphingosine) might serve to restore the synthesis of sphingolipids. We also tested Aur-A (aureobasidin A), an inhibitor of IPC synthase [14]. We provided evidence showing that IPC synthesis was required for the generation of DAG during the presumptive step of PKC activation and for the normal transit through the cell cycle. We found that PHS restored IPC synthesis and cell cycle progression in cells in which SPT was inhibited, and that IPC synthesis was required for the generation of DAG. We discuss the interesting possibility that IPC synthesis could account not only for the control of the ceramide levels, but also in DAG generation. During the synthesis of IPCs, DAG was generated, and the amounts of ceramide and sphingoid bases were decreased. These are adequate conditions for the activation of the putative PKC.

MATERIALS AND METHODS

Materials

^{32}P (carrier-free; 10 mCi/ml), [γ-^{32}P]ATP (PB-108; > 5000 Ci/mmol), *myo*-[2-^{3}H]inositol (10–20 Ci/mmol), [9,10(n)-^{3}H]palmitic acid (40–60 Ci/mmol) and DAG assay system were purchased from Amersham Biosciences. Peptone and yeast extract were purchased from Difco Laboratories (Detroit, MI, U.S.A.), the synthetic medium was from Q-biogene (Vista, CA, U.S.A.), β-chloroalanine, *o*-phthalaldehyde, myriocin and other chemicals were from Sigma, Aur-A was from PanVera (Madison, WI, U.S.A.), organic solvents and Silica gel 60 thin layer plates were obtained from Merck (México) and scintillation liquid from New England Nuclear (Perkin Elmer, México). Phospholipids and neutral lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and Sigma.

Strains and culture conditions

S. cerevisiae A 364 A (*Mata, ade1, ade2, ura1, his7, lys2, tyr1, gal1*) obtained from the Department of Genetics (CINVESTAV-IPN, Mexico) and *S. cerevisiae* ATCC 9080 (A.T.C.C., Manassas, VA, U.S.A.), a natural inositol auxothroph, were used in the experiments. Cultures were maintained on YPD medium [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose, which was supplemented with 50 mM succinic acid] containing 1.5 % (w/v) bacto-agar. Strains were propagated in CSM (complete synthetic medium) and Molecular Biology Certified yeast growth media (Q-biogene), containing 2 % glucose, 6.7 g/l nitrogen base, 0.8 g/l supplement mixture (nucleotides and amino acids), 5 g/l ammonium sulphate and 2 μg/ml inositol. Cultures were performed in a rotary shaker (160 rev./min at 30 °C) and harvested in the stationary phase of growth. These cells were used in turn as the inoculum for each experiment (G_0/G_1 cells). For nutrient induction experiments, cells were grown in CSM to stationary phase (24 h), and transferred to starvation media by centrifugation and resuspension in N_2-starvation medium MM-N (minimal medium without nitrogen), which contained yeast nitrogen base, 2.0 % glucose and 2 μg/ml inositol, without ammonium sulphate and amino acids at A_{600} and incubated for 16–20 h at 160 rev./min at 30 °C. The cell concentration and percentage of budded cells were determined. At least 200 cells were scored for their bud morphology. Microscopic examination and plating experiments indicated an arrest of 90–95 % unbudded (G_1) viable cells. These cells were washed twice with MM-N and were used as the inoculum for the study of the G_1 to S transition.

Growth re-initiation

Cells were incubated in CSM at 30 °C in the absence and presence of the indicated concentrations of the inhibitors of sphingolipid synthesis. Myriocin and Aur-A were dissolved in ethanol and added to CSM to the final concentrations as indicated in the Results section. The concentration of ethanol in CSM was 0.5 %. β-chloroalanine dissolved in water. Proliferation was determined as the absorbance *A* at 600 nm. For viability measurement, an yeast culture was mixed with an equal volume of a Methylene Blue solution (0.2 mg/ml in 0.1 M phosphate buffer, pH 4.6). The mixture was left for 5 min at room temperature (23 °C) and total cells and stained cells (dead) were counted under a microscope. Viability was expressed as the mean of the percentage of stained cells.

Labelling and analysis of sphingolipids, phospholipids and neutral lipids

Pulse and steady-state labelling of lipids with *myo*-[2-^{3}H]inositol, [9,10(n)-^{3}H]palmitic acid and [^{32}P]P_i were performed to quantify the effects of inhibitors on total sphingolipid synthesis. Washed G_0/G_1 cells were resuspended to an initial density of approx. 10^6 cells/ml in CSM in the absence or presence of the inhibitor as indicated; cells were then incubated with the radiolabelled precursor for the indicated time periods to pulse-label sphingolipids, phospholipids and neutral lipids. Lipids were extracted from labelled cells using a method described by Hanson and Lester [15]. Cells were treated with 5 % (w/v) (final concentration) trichloroacetic acid for 20 min at 4 °C. Cells were centrifuged, washed twice with 1.0 ml of 5 % trichloroacetic acid, and finally with water. Cell pellets were extracted with 1.0 ml of 95 % ethanol/water/diethyl ether/pyridine (15:15:5:1, by vol.) for 60 min at 60 °C. Phospholipids were analysed by one-dimensional TLC on silica gel plates activated at 110 °C for 15 min and impregnated with 1 % potassium oxalate and 2 mM NaEDTA in methanol/water (2:3, v/v). TLC was developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.). In some experiments, sphingolipids were analysed after alkaline hydrolysis of glycerophospholipids. Lipids were deacylated by the addition of 1 ml of 0.2 M NaOH in methanol and on incubation at 30 °C for 45–60 min. After the addition of 1 ml of 5 % EDTA, samples were neutralized by the addition of 0.2 ml of 1 M acetic acid (60 g/l). Lipids were then extracted with 1 ml of chloroform, dried under N_2, resuspended in 250 μl of chloroform and 50 μl samples were analysed by TLC. Neutral lipids were separated by one-dimensional TLC on silica gel G plates, developed with light petroleum (b.p. 30–75 °C)/ethyl ether/acetic acid (90:10:1, by vol.). After separation, lipids were visualized by exposure of the plates to iodine vapour or after autoradiography of plates, where the corresponding standards were included. The individual phospholipids were removed from the plate and quantified by scintillation counting. The phosphorus in phospholipids (nmol of P_i present in the chloroform phase) was determined by the method of Ames and Dubin [16]. The resultant phosphate value was used to normalize samples against each other (see [17]). Radioactivity

was expressed in terms of c.p.m. or d.p.m. in 10^7 cells. Inositol-containing sphingolipids were identified based on their non-saponifiable characteristic. When the [*myo*-2-^{3}H]inositol- and [^{32}P]P_i-labelled non-deacylable lipid fractions were subjected to TLC, 4–5 bands were found and R_F values were 0.5, 0.44, 0.40, 0.37 and 0.28. Two of these lipids (R_F 0.4 and 0.28) gave a positive test when sprayed with the orcinol-sulphuric acid reagent and were labelled with [^{14}C]mannose (60 min pulse), indicating that they were MIPC and $M(IP)_2C$. The upper one (R_F 0.4) does not separate well by TLC and labelling of IPC + MIPC was evaluated. The lower one (R_F 0.28), in addition to being more polar, was identified as $M(IP)_2C$, since pulse labelling shows that the R_F 0.28 band was labelled in relation to their precursors (IPC and MIPC). Quantification was performed in the same TLC plate. The restricted number of manipulations avoids loss of the sample and inaccuracies. The chromatographic mobilities observed compare well with the results reported by Smith and Lester [18]. Also, after alkaline hydrolysis, the amide bond of ceramides was detected by infrared spectroscopy [19]. Glycerophospholipids were identified by co-migration with standards from Sigma and Avanti Polar. Also, phosphatidylethanolamine and PS (phosphatidylserine) were identified by trinitrobenzylation, PI by incorporation of [^{3}H]inositol and PC by incorporation of [^{3}H]methyl choline as described previously [20].

DAG mass measurements

A radioenzymatic assay employing *Escherichia coli* DAG kinase (Amersham Biosciences) was used for a quantitative determination of DAG under defined mixed micelle conditions to solubilize the DAG. The enzyme converts DAG to [^{32}P]phosphatidic acid with [γ-^{32}P]ATP (PB 108; > 5000 Ci/mmol). The assay was performed according to the manufacturer's instructions. [^{32}P]Phosphatidic acid was extracted and subsequently separated from other lipids and residual [γ-^{32}P]ATP by TLC, using phosphatidic acid (Sigma) as a reference. The radioactivity of [^{32}P]phosphatidic acid was estimated by liquid scintillation counting. DAG/phospholipid ratios were calculated and expressed as pmol of DAG/nmol of phosphate. The use of DAG/phospholipid ratios corrects for the differences in cell number and recoveries. Ceramide levels were evaluated also using the DAG kinase method using ceramide phosphate (Sigma) as described previously [21].

Measurement of levels of sphingoid bases

Sphingoid bases from unlabelled cells were quantified using HPLC as described by Merrill et al. [22]. Sphingoid base standards were used for the identification and quantification. Initial studies indicated that a minimum of 1×10^7 yeast cells were required for sufficient cellular sphingoid bases to be measured by this method.

Measurement of phospholipid turnover

G_0/G_1 cells were subcultured in CSM containing [^{3}H]palmitic acid (5 μCi/ml) in the absence or presence of 5 mM β-chloroalanine. At time zero, the average cell number was $(1.0–1.2) \times 10^6$ cells/ml. After 20 min, the cells were washed with PBS, and fresh medium in the absence or presence of 5 mM β-chloroalanine was added. At the indicated times, up until 4 h, duplicate samples were treated with trichloroacetic acid, and lipids were extracted, separated by TLC and quantified. At the 20 min pulse time, there was $(146 \pm 10) \times 10^3$ d.p.m./10^7 cells in the control cells and $(140 \pm 12) \times 10^3$ d.p.m./10^7 cells in the β-chloroalanine-treated cells. At the indicated times, duplicate samples were harvested, and lipids extracted and analysed as described above.

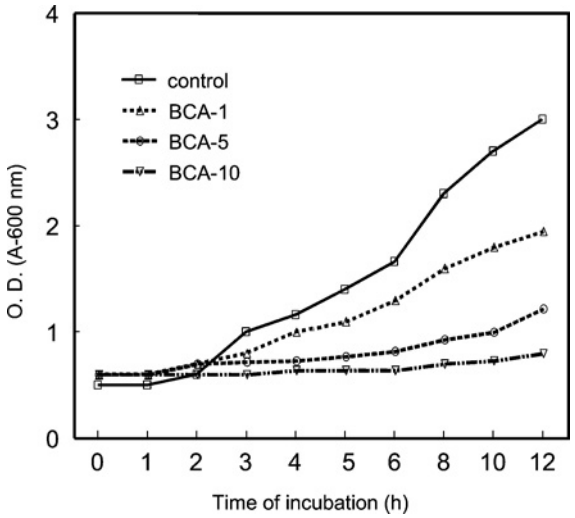

Figure 1 Effects of β-chloroalanine on yeast cell proliferation

Yeast cultures were initiated in CSM (A_{600} 0.5 unit) from 24 h cultures (G_0/G_1 cells). The indicated concentrations of β-chloroalanine (BCA) were added (0, 1, 5 and 10 mM). Growth was determined at the indicated time points after the addition of β-chloroalanine by measurement of A_{600} of the culture. The results shown are representative of two independent experiments.

RESULTS

Effects of β-chloroalanine on yeast cell viability and proliferation

Inhibitors of specific steps in yeast and mammalian sphingolipid biosynthesis have been identified and proved to be useful in understanding sphingolipid functions. β-Chloroalanine and myriocin (ISP-1) inhibit SPT, and it has been suggested that the first step in sphingolipid biosynthesis is probably regulated and found to be important for controlling the rate of ceramide synthesis *de novo* and the rate of complex sphingolipid synthesis [23]. Figure 1 shows that there was a concentration-dependent inhibition of cell proliferation and that 10 mM β-chloroalanine resulted in 90 % inhibition. No lysis or other gross changes in cell morphology were observed in drug-treated cells and cells were able to grow after washing out the β-chloroalanine even after 10 h of exposure. There was no significant loss of cell viability (5–7.5 % dead cells). This means that β-chloroalanine-treated cells were prevented from normal cell division for an extended period of time. The response to β-chloroalanine was decreased by increasing cell number; thus all subsequent experiments were conducted at starting cell concentrations of 0.2–0.5 absorbance unit/ml.

Effects of β-chloroalanine on phospholipid synthesis

Exogenously added [^{32}P]P_i was used to estimate the synthesis of phospholipids. G_0/G_1 cells were washed twice with 25 ml of water and then were resuspended to an A_{600} of 0.4 in 20 ml of CSM [$(4.0–4.4) \times 10^7$ cells] and labelled with 5 μCi/ml [^{32}P]P_i for 60 min in the absence or presence of 5 mM β-chloroalanine. In Figure 2, the results of TLC and autoradiography (top panel) and the radioactivity incorporated into lipids (bottom panel) show that β-chloroalanine decreased the amount of ^{32}P in sphingolipids to 53 % of the control value ($P < 0.001$), whereas the amount of label incorporated into the precursor PI was increased slightly. Although there was a 24 % decrement in the amount of ^{32}P incorporated into PC ($P < 0.05$), the incorporation of radiolabel into total lipid was not altered by exposure to β-chloroalanine during this period of time [$(298 \pm 15) \times 10^3$ compared with $(263 \pm 21) \times 10^3$ c.p.m./10^7 cells]. Similar results were obtained with the

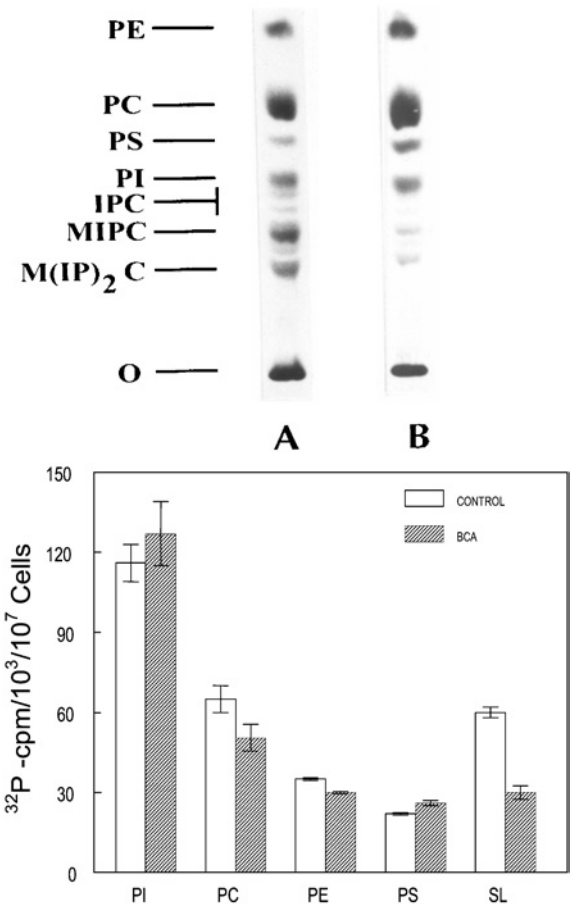

Figure 2 Effects of β-chloroalanine on phospholipid synthesis

Cultures (20 ml; A_{600} 0.4 unit/ml) of G_0/G_1 [$(4.0–4.4) \times 10^7$] cells were radiolabelled with 5 μCi/ml [^{32}P]P_i during a 60 min pulse. Lipids were extracted and subjected to TLC and autoradiography [top panel: lane A, control cells; lane B, cells exposed to 5 mM β-chloroalanine (BCA)]. Individual phospholipids were quantified by scraping off the radioactive spots and estimating the radioactivity by scintillation counting (lower panel). Values represent the means $\pm$ S.D. of triplicate samples from a representative experiment. Migration of standards is indicated, and phosphatidylethanolamine (PE), PC, PS, PI and IPC/MIPC and M(IP)$_2$C were identified as described in the Materials and methods section. Note the inhibition of [^{32}P]P_i incorporation into sphingolipids by β-chloroalanine. Student's t test was used to determine the significance of differences in phospholipid synthesis in control compared with β-chloroalanine-exposed cells: PC, $P < 0.05$; sphingolipid (SL), $P < 0.001$.

inositol auxothroph strain (A.T.C.C. 9080). After 60 min of incubation in the presence of 5 mM β-chloroalanine, sphingolipid labelling was decreased to 58 % of control [from $(99 \pm 10) \times 10^3$ to $(53 \pm 15) \times 10^3$ c.p.m./10^7 cells; $P < 0.01$], whereas the amount of labelled PI was increased slightly [from $(98 \pm 6) \times 10^3$ to $(116 \pm 17) \times 10^3$ c.p.m./10^7 cells]. Also, the label incorporated into PC was decreased by 23 %.

Effects of time of incubation and β-chloroalanine on cellular DAG generation

G_0/G_1 cells were pre-labelled with [^{3}H]palmitic acid (1 μCi/ml) to steady state (during 24 h), washed twice with water and resuspended to an absorbance of 0.2 unit/ml in CSM without radiolabel in the absence or presence of 5 mM β-chloroalanine. At the indicated times, lipids were extracted, neutral lipids were separated by TLC and the label incorporated in DAG was determined. Figure 3 shows that labelled DAG was increased 2.2 times during the first 40 min of incubation, and then decreased towards the initial values

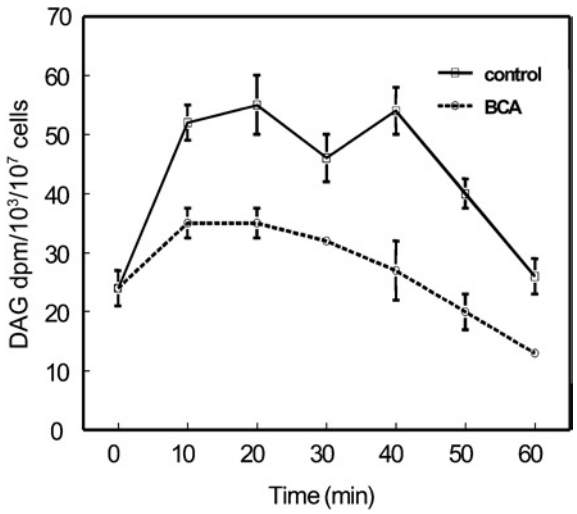

Figure 3 Influence of time of incubation and β-chloroalanine on DAG levels

Yeast cells were prelabelled with [^{3}H]palmitic acid (1 μCi/ml) for 24 h, washed, and $(4.0–4.4) \times 10^7$ cells were resuspended in 20 ml of CSM and incubated at 30 °C. At the indicated times, neutral lipids were extracted, separated by TLC and [^{3}H]DAG was quantified. Values are expressed as d.p.m./10^3 in 10^7 cells and are means $\pm$ S.D. of triplicate samples from a representative experiment. Note the inhibition of [^{3}H]DAG generation by β-chloroalanine (BCA). Significance of differences in control compared with treated cells: 20, 40 and 50 min, $^*P < 0.01$; 30 min, $P < 0.05$.

after 60 min of incubation. In the cells exposed to β-chloroalanine, after a transitory increment during the first 10–20 min, levels of labelled DAG were decreased to 0.5–0.3 times the control levels at 40 min of incubation ($P < 0.01$). The inhibition of both the generation of DAG and sphingolipid synthesis by β-chloroalanine suggested that these two processes are probably related.

Effects of β-chloroalanine on phospholipid turnover

Previous studies on the dynamics of inositol-containing phospholipids in *S. cerevisiae* during growth [24], showed that during the lag phase (first 2 h of incubation) there was a significant increment of 9.0–21.6 % in labelled IPCs (IPC + MIPC) accompanied by a 16 % decrement in PI in [^{3}H]inositol pre-labelled yeast cells at steady state. Therefore we examined the effects of 5 mM β-chloroalanine on inositol phospholipid turnover using a 20 min pulse with [^{3}H]palmitic acid (5 μCi/ml) in the absence or presence of 5 mM β-chloroalanine, and a 1–4 h chase. After the 20 min pulse, the incorporation of radiolabel into total phospholipids was similar in both the control and β-chloroalanine-treated cells [$(146 \pm 10) \times 10^3$ and $(140 \pm 12) \times 10^3$ d.p.m./10^7 cells respectively]. Figure 4 shows that labelled PI was decreased to 54 % after a 60 min chase, whereas labelled IPCs were increased 1.44 times. After a 120–240 min chase, labelled PI was further decreased (75 %) whereas labelled IPCs were increased 2.2 times. In the β-chloroalanine-treated cells, there were no significant changes in labelled IPCs and labelled PI was not decreased during the first 2 h of chase. These results suggest that the slightly increased labelling of PI observed in the β-chloroalanine-treated cells (Figure 2) was due to the inhibition of IPC synthesis and that there was no detectable degradation of sphingolipids.

Changes in endogenous sphingoid bases during growth initiation

To ensure that β-chloroalanine addition had effectively inhibited the activity of SPT, we determined the levels of DHS (D-erythrosphingosine) and PHS during growth re-initiation. It can

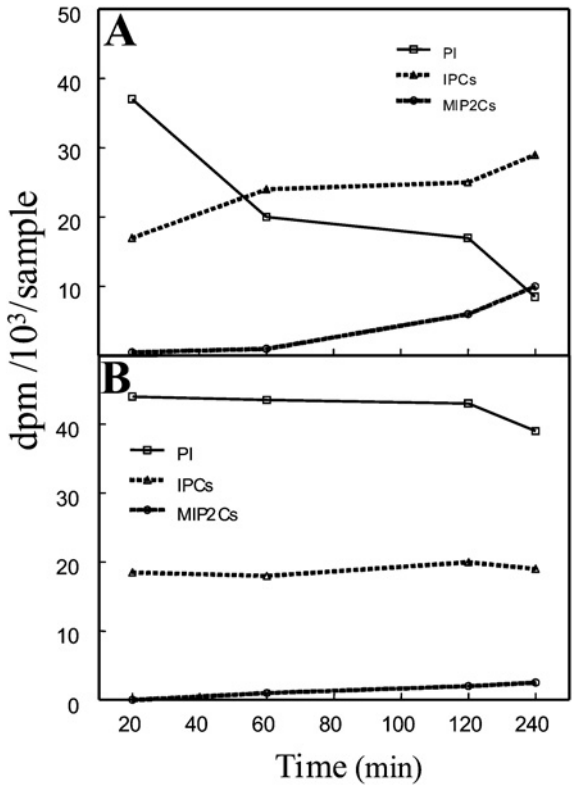

Figure 4 Effects of 5 mM β-chloroalanine on phospholipid turnover

G_0/G_1 yeast cells [(1.0–1.2) $\times$ 10^6 cells/ml] were seeded in CSM and pulse-labelled for 20 min with 5 μCi/ml [^{3}H]palmitic acid in the absence or presence of 5 mM β-chloroalanine. After 20 min, the cells were washed twice with CSM, followed by culture medium without isotope, with (**B**) or without (**A**) 5 mM β-chloroalanine. At the indicated times, [^{3}H]palmitic acid incorporated into PI, IPC/MIPC and $M(IP)_2C$ was quantified. Results are expressed as d.p.m./10^3 in 10^7 cells. Values represent the results of one of three independent experiments, all of which gave essentially identical results.

Table 1 Cellular concentrations of sphingoid bases during yeast proliferation

G_0/G_1 yeast cells were incubated in CSM at 30 °C. At the indicated times, sphingoid bases were extracted and their *o*-phthaldehyde derivatives were prepared and analysed by HPLC as described in the text. Values are means $\pm$ S.D. for a representative experiment performed in triplicate. ND, not detected.

		Sphingoid base (pmol/10^7 cells)	
Growth phase	Time (h)	DHS	PHS
Lag	0	43.3 $\pm$ 7.0	9.3 $\pm$ 1.4
	2	1.0 $\pm$ 0.3	12.0 $\pm$ 2.0
Exponential	4	0.72 $\pm$ 0.14	17.0 $\pm$ 2.0
	6	ND	1.7 $\pm$ 0.5
	8	1.3 $\pm$ 0.2	0.8 $\pm$ 0.16
Late exponential	10	3.0 $\pm$ 1.0	2.3 $\pm$ 1.0
	12	19.6 $\pm$ 2.0	10.6 $\pm$ 1.5
Stationary	24	42.0 $\pm$ 7.0	9.3 $\pm$ 1.4

be seen (Table 1) that after 2 h of incubation in CSM (lag phase), the levels of sphingoid bases were decreased to 25 % of the levels observed at 0 h. After 8 h of incubation (exponential phase), only 2.5 % of sphingoid base levels determined at 0 h remained, indicating an efficient metabolic transformation following growth re-initiation. During the late-exponential to stationary phase of growth (12–24 h), the levels of DHS and PHS were increased towards the initial values. In the cells exposed to β-chloroalanine, after 2 h of incubation, DHS and PHS were not detected, which is consistent with inhibition of the synthesis of sphingoid bases. The inhibition by long-chain bases of PKC and other enzymes, including CTP:phosphocholine transferase [25], IPC synthase, PS synthase and phosphatidate phosphohydrolase, was observed *in vitro*, and suggested to occur *in vivo* [26]. However, this possibility seems unlikely during the lag–exponential phases of growth, considering the decreased concentrations of DHS and PHS determined. These results also suggested that the decrease in the synthesis of PC observed in the β-chloroalanine-treated cells could be related to the decreased generation of DAG and not to the inhibition by sphingoid bases of the CTP:phosphocholine transferase.

Table 2 Effects of β-chloroalanine (BCA), myriocin (ISP-1) and Aur-A (inhibitors of the synthesis of sphingolipids) on the G_1 to S transition

After N_2 starvation (90–95 % unbudded cells) G_1 yeast cells were resuspended in CSM and incubated at 30 °C in the absence (control) or presence of inhibitor, and at the indicated times after the release of N_2 starvation the percentage of budded cells was determined by microscopic examination.

	Budded cells (%)				
Treatment	0 min	60 min	120 min	180 min	Inhibition (%)
Control	8	18	43	73	0
BCA (5 mM)	8	13	19	22	70
Myriocin					
5 μM	8	16	29	33	55
20 μM	8	12	20	23	69
Aur-A					
5 μM	7	7	18	36	51
20 μM	5	5	9	18	75
Myriocin (20 μM) + PHS (50 μM)	8	13	26	59	20

Effects of inhibiting the synthesis of sphingolipids on the G_1 to S transition

It has been reported that there was a 2–3-fold increase in DAG levels prior to budding in cells re-entering the cell cycle following recovery from G_1 arrest, induced by mating pheromone as well as in early G_1 cells obtained by centrifugal elutriation [4]. Having found that β-chloroalanine inhibited sphingolipid synthesis, decreased the DAG levels and inhibited proliferation, we tested the effect of β-chloroalanine on the G_1 to S transition in G_1 cells synchronized by nitrogen arrest. Since β-chloroalanine appears to inhibit various pyridoxal phosphate-dependent enzymes, in addition to SPT, we examined if myriocin, another specific inhibitor of SPT [14] added to the incubation medium, inhibits the G_1 to S transition. We also tested the effects of Aur-A, an inhibitor of IPC synthase, considering that DAG is generated during the synthesis of IPC. The results in Table 2 show the effects of inhibiting the synthesis of sphingolipids on the G_1 to S transition. After release of N_2 starvation (180 min of incubation time), the G_1 to S transition (budding) was inhibited to 70 % with 5 mM β-chloroalanine, 55–69 % with myriocin (10–20 μM) and 51–75 % with Aur-A (4.5–20 μM). Inhibition of the G_1 to S transition with 20 μM myriocin was restored by metabolic complementation with 50 μM PHS, indicating that the inhibition of SPT was specific. When the synthesis of sphingolipids was determined after N_2 starvation, it was found that [^{3}H]palmitic acid (3 μCi/ml) labelling

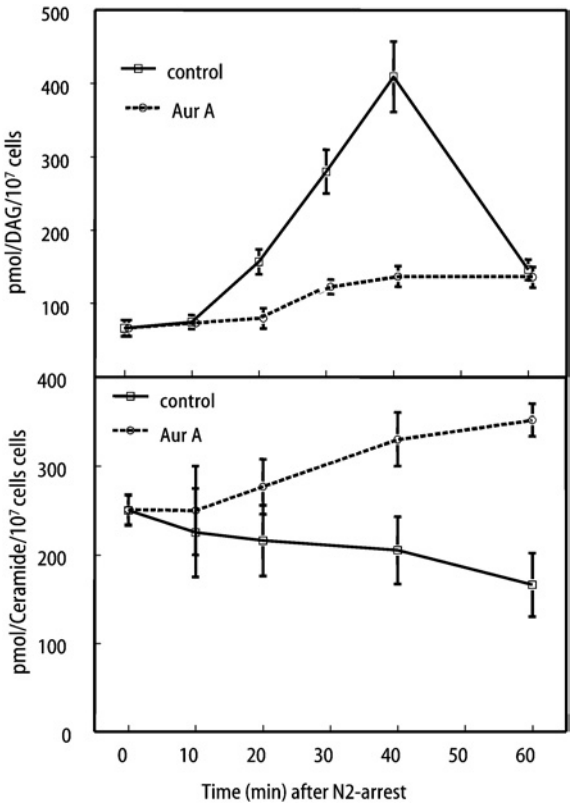

Figure 5 Effects of time of incubation and inhibition of IPC synthesis by Aur-A on cellular DAG and ceramide levels analysed by mass measurements

After N_2-induced arrest, G_1 yeast cells were seeded in CSM at a cell density of (2.4–2.6) $\times 10^7$ cells/10 ml in the absence or presence of 20 μM Aur-A and, at the indicated times, analysed for DAG, ceramide content and total phospholipid phosphorus as described in the text. The results are expressed in pmol/10^7 cells after correction for the amount of phospholipid phosphorus in the sample. Results are means $\pm$ S.D. for triplicate determinations from a representative experiment. Note the decrements in the levels of DAG during the first 40 min of incubation and increments in ceramide in the cells exposed to Aur-A ($P < 0.01$).

of sphingolipids during a 60 min pulse was decreased to 29 % of control with 5 mM β-chloroalanine, to 39 % with 20 μM myriocin and to 34 % with 20 μM Aur-A.

Effects of Aur-A on cellular concentrations of ceramide and DAG mass levels during the G_1 to S transition

IPC synthase transfers the phosphoinositol head group from PI to ceramide, forming IPC and generating DAG. This activity places IPC synthase at an important regulatory position in signalling pathways that utilize ceramide and DAG as second messengers, because it could act to switch a growth arrest to a mitogenic signal. Therefore we decided to determine the balance between mitogenic DAG and anti-mitogenic ceramide during the G_1 to S transition and the effect of the inhibition of IPC synthase with Aur-A. Figure 5 shows that during the first 40 min of incubation in CSM after release of N_2 starvation, the amount of DAG in the control non-exposed cells was increased 6 times and decreased towards the initial values after 60 min of incubation and ceramide levels were decreased by 1.5 times. During the first 40 min of incubation in the cells exposed to Aur-A, the amount of DAG was decreased to 33 % of control values ($P < 0.01$) and during the first 60 min of incubation, ceramide levels were increased 1.4 times ($P < 0.01$). The DAG/ceramide ratio was high in proliferating cells (2.0 versus 0.4 in the cells arrested in G_1 with Aur-A), indicating that IPC

Table 3 Cellular concentrations of sphingoid bases after release of N_2 starvation (G_1 to S transition)

G_1 cells were incubated in CSM at 30 °C and, at the indicated times, sphingoid bases were extracted and their *o*-phtalaldehyde derivatives were prepared and analysed by HPLC as described in the text. Values are means $\pm$ S.D. for four separate experiments.

	Sphingoid base (pmol/10^7 cells)		
Time (min) after N_2-starvation	DHS	PHS	Budded cells (%)
0	48 $\pm$ 6	32 $\pm$ 2	7.2
20	24 $\pm$ 1.5	19 $\pm$ 0.5	6.8
40	14 $\pm$ 1.25	2.8 $\pm$ 1.5	8.0
60	0.2 $\pm$ 0.05	0.4 $\pm$ 0.3	10.4

synthesis, DAG generation and decreased levels of ceramide were required for the normal transit through the cell cycle.

Changes in sphingoid base levels during the G_1 to S transition

To determine whether the increments in DAG and the decrements in ceramide coincided with decrements in DHS and PHS, the levels of sphingoid bases were determined. Table 3 shows that, after the release of N_2-starvation, the levels of sphingoid bases were decreased to 53 % after 20 min and to 21 % after 40 min; after 60 min, only 0.75 % of the levels remained compared with those observed at 0 h. These results, together with those of Table 1, indicate that sphingolipid metabolism was efficiently co-ordinated, and that preformed ceramide, DHS and PHS were metabolized during growth re-initiation. To know if these precursors were used for the synthesis of IPCs, yeast cells prelabelled with [^{3}H]inositol (1 μCi/ml) during a 24 h period were N_2-starved, and G_1 cells (90–95 % unbudded cells) were washed and resuspended in CSM at 30 °C. At the indicated times, lipids were extracted, separated by TLC and radioactivity incorporated in IPC/MIPC and $M(IP)_2C$ was determined. Figure 6 shows that there was a significant increment of [^{3}H]inositol radioactivity incorporated in sphingolipids. During the first 60 min, a 2.4-fold increment was observed [76 % in IPC/MIPC and 24 % in $M(IP)_2C$], and between 60 and 120 min the radioactivity incorporated was increased 1.5 times more [61 % in IPC/MIPC and 39 % in $M(IP)_2C$]. Radioactivity of the prelabelled [^{3}H]PI was decreased 1.8 times between 0 and 2 h, indicating that in addition to synthesis *de novo*, the preformed precursors DHS, PHS, ceramide and PI were used for the synthesis of sphingolipids. Therefore the conditions of increased DAG levels, together with decreased levels of ceramide and sphingoid bases, were adequate for the activation of the putative PKC necessary for the G_1 to S transition and cell proliferation.

Rate of phospholipid synthesis

Having found that during the first 20–40 min of incubation, the amount of DAG was increased (Figure 3), and in order to determine if all the DAG was generated during the synthesis of IPCs, the rate of phospholipid synthesis and DAG mass levels were determined. G_0/G_1 yeast cells were incubated in CSM containing 20 μCi/ml [^{32}P]P_i for 30–60 min and phospholipid labelling was determined. From the knowledge of the specific radioactivity of [^{32}P]P_i in the culture medium and from the absorbance of the culture at the end of the pulse period, the rate of incorporation was calculated. It was found that during the first 30 min of incubation, the rate of phospholipid synthesis was 1450 $\pm$ 100 pmol/10^7 cells and after 60 min it was 2636 $\pm$ 300 pmol/10^7 cells. Considering that 20–23 % of the radioactivity was in the sphingolipids,

[^{3}H]-inositol dpm/10^7 cells			
time (min)	PI	IPC/MIPC	M(IP)2C
0	33684	2639	391
60	24046	5700	1500
120	18463	7259	4594

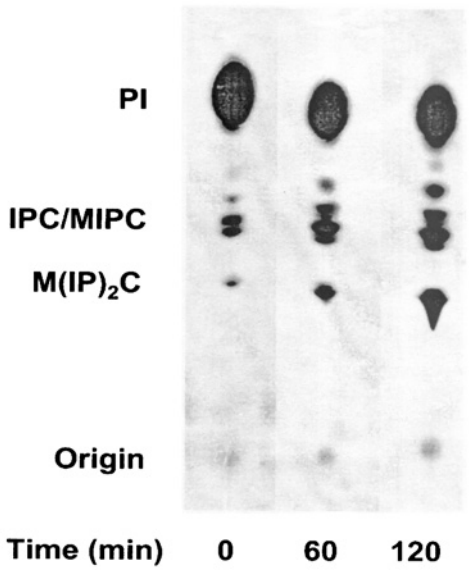

Figure 6 Preformed PI is used for the synthesis of IPCs during the G_1 to S transition

Yeast cells were prelabelled with [^{3}H]inositol (1 μCi/ml) during a 24 h period, N_2-starved and (4.0–4.4) $\times$ 10^7 labelled G_1 cells (90–95 % unbudded cells) were seeded in 20 ml of CSM. At the indicated times, the radioactivity incorporated into PI, IPC/MIPC and M(IP)$_2$C was determined. The results are expressed as d.p.m./10^7 cells. IPC/MIPC and M(IP)$_2$C were identified as described in the Materials and methods section.

290–323 and 535–606 pmol of IPC/10^7 cells was synthesized during the first 30 and 60 min respectively of incubation. The DAG mass levels were 300 ± 30 and 350 ± 50 pmol/10^7 cells respectively, and considering that 1 mol of DAG was generated for every 1 mol of IPC synthesized, the synthesis of sphingolipids was sufficient to account for the DAG generated during growth re-initiation. A similar amount of DAG (409 ± 48 pmol/10^7 cells) was determined during the first 40 min of incubation after the release of N_2 arrest (Figure 5).

DISCUSSION

During growth re-initiation (G_0/G_1cells), a clear correlation between sphingolipid synthesis (Figure 2) and DAG generation (Figure 3) was observed in coincidence with a decrement in DHS and PHS (Table 1). We also found that the inhibition of sphingolipid synthesis with β-chloroalanine decreased the generation of DAG, suggesting that these two processes were related. The study of G_1 cells showed that all these processes take place during the G_1 to S transition. The decrease in sphingolipid synthesis with β-chloroalanine and myriocin, inhibitors of SPT, as well as the inhibition of IPC synthase with Aur-A, blocked the G_1 to S transition (Table 2) and DAG generation (Figure 5). The restoration of the G_1 to S transition by the addition of PHS indicated that SPT inhibition by myriocin was specific. We previously observed that addition of DHS to β-chloroalanine-exposed Madin–Darby canine kidney cells restored both the generation of DAG and cell proliferation [27] and it has been reported that PHS restored growth of myriocin-exposed *S. cerevisiae* cells [28]. In *S. cerevisiae* cells during growth re-initiation, long-chain bases and ceramide levels were decreased (Tables 1 and 3 and Figure 5), whereas the generation of DAG was increased (Figures 3 and 5), and these are adequate conditions for the activation of the putative PKC. The role of DAG in the activation of Pkc-1p remains a controversial point. Thus, in contrast to the mammalian system, *in vitro* Pkc-1p was completely inactive, and a partially purified Pkc-1p was independent of cofactors (Ca^{2+}, DAG and PS) [29]. Nevertheless, it has been found that the sites for binding of DAG are highly conserved, and the study of mutants affected in the putative DAG-binding site of *S. cerevisiae* PKC indicates that DAG may regulate Pkc-1p activity [30]. In addition, increased DAG levels have been found in a yeast Cdc 28 mutant during the G_1 to S transition [4] and we found that the DAG generated during sphingomyelin synthesis is involved in PKC activation in Madin–Darby canine kidney cells [27]. Also, human PKC-ε was able to complement the growth defect caused by deletion of PKC-1 in *S. cerevisiae*, and PKC-ε is DAG-dependent [31]. Therefore it is possible that suitable *in vitro* conditions that determine PKC-1 activity in *S. cerevisiae* extracts have not yet been found. Considering all the above-mentioned results, it seems that DAG generation during the synthesis of sphingolipids is a prerequisite for the transit of yeast cells through the cell cycle and probably of PKC-1 activation and proliferation. The contribution of IPC synthesis to the generation of DAG was also shown when the synthesis was inhibited at the site of IPC synthase with Aur-A (Figure 5). *S. cerevisiae* cells have been shown to display a cell cycle-dependent pathway involving the hydrolysis of PC to DAG and choline phosphate during the G_1 to S transition [4]. However, it is unlikely that the concentrations of β-chloroalanine, myriocin and Aur-A that decreased the levels of DAG were inhibiting PC-phospholipase C. Considering that the increments observed in choline phosphate coincided with decrements in labelled glycerophosphocholine [4] and that PC must be turned over for the synthesis of PC in the absence of external choline [32], it seems that the increase in labelled choline phosphate might be related to PC turnover.

The accumulation of DHS and PHS during the late exponential phase and ceramide during the stationary phase of growth may participate in the processes that cause yeast cells to enter and remain in the stationary phase of growth, and deserve further investigation. All the above-mentioned results suggested that sphingolipids act as signalling molecules in *S. cerevisiae* during unstressed conditions also, and this signalling process might be preserved from yeast to mammals. The observations reported in the present and the previous studies [27] indicate that sphingolipid and glycerolipid metabolism and signalling are coupled or interrelated. A perturbation in the DAG/ceramide ratio may lead to changes in cell growth and viability. Under growth-inhibited conditions (N_2-arrested cells), the DAG/ceramide ratio was 0.26, and it changed to 2.0 during growth re-initiation (first 40 min). To our knowledge, this is the first evidence indicating that DAG generated during IPC synthesis participates in signalling and probably PKC activation. The pool of DAG analysed is derived from PI but not for activation of catabolic pathways. Considering that there is no evidence for degradation of IPC, MIPC and M(IP)$_2$C in *S. cerevisiae* [33] (for a review see [33]), the observed accumulation of bioactive intermediates can be considered as being due to sphingolipid synthesis *de novo*. The role of ceramide, sphingoid bases and their derivatives under stressed conditions has been reviewed [23,33–35]. A paper that appeared just as the present study was completed showed that down-regulation of IPC synthase decreased DAG levels, and that the recombinant Pkc-1 protein of

Cryptococcus neoformans transformed in *S. cerevisiae* was activated by DAG [36].

We thank Dr A. Garcia-Sainz and Dr J. L. Reyes for critically reviewing the manuscript. This work was supported by a Consejo Nacional de Ciencia y Tecnologia (CONACYT) grant 211085-5-26404 and fellowships to D.S.-C. and A.F.

REFERENCES

1 Nickeis, J. T. and Broach, J. R. (1996) A ceramide-activated protein phosphatase mediates ceramide-induced G_1 arrest of *Saccharomyces cerevisiae*. Genes Dev. **10**, 382–394
2 Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garret, S. and Hannun, Y. A. (1993) Ceramide-mediated growth inhibition and CAPP are conserved in *Saccharomyces cerevisiae*. J. Biol. Chem. **268**, 9255–9261
3 Nagiec, N. M., Nagiec, E. E., Baltisberger, J. A., Wells, G. B., Lester, R. L. and Dickson, R. C. (1997) Sphingolipid synthesis as a target for antifungal drugs – complementation of the inositol phosphoryl ceramide synthase defect in a mutant strain of *Sacharomyces cerevisiae* by the Aur 1 gene. J. Biol. Chem. **272**, 9809–9817
4 Marini, M. J., Meldrum, E., Buehrer, B., Hubberstey, A. V., Stone, D. E., Traynor-Kaplan, A. and Reed, S. I. (1996) A pathway in the yeast cell division cycle linking protein kinase C (Pkc 1) to activation of Cdc 28 at start. EMBO J. **15**, 3040–3052
5 Berridge, M. J. and Irvine, R. F. (1989) Inositol phosphate and cell signaling. Nature (London) **341**, 197–205
6 Schomerus, Ch. and Kuntzel, H. (1992) CDC 25-dependent induction of inositol 1,4,5 triphosphate and diacylglycerol in *Saccharomyces cerevisiae* by nitrogen. FEBS Lett. **307**, 249–252
7 Hawkins, P. T., Stephens, L. R. and Piggott, J. R. (1993) Analysis of inositol metabolites produced by *Saccharomyces cerevisiae* in response to glucose stimulation. J. Biol. Chem. **268**, 3374–3383
8 Mayr, J. A., Kohlwein, S. D. and Paltauf, F. (1996) Identification of a novel Ca^{2+}-dependent phospholipase D with preference for phosphatidylserine and phosphatidylethanolamine in *Saccharomyces cerevisiae*. FEBS Lett. **393**, 236–240
9 Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W. I., Voelker, D. R. and Carman, G. M. (1998) Isolation and characterization of *Saccharomyces cerevisiae* LPP1 gene encoding a Mg^{2+} independent phosphatidate phosphatase. J. Biol. Chem. **273**, 14331–14338
10 McGraw, P. and Henry, S. A. (1989) Mutations in the *Saccharomyces cerevisiae* Opi 3 gene: effects on phospholipid methylation, growth and cross-pathway regulation of inositol synthesis. Genetics **122**, 317–330
11 Wells, G. B. and Lester, R. L. (1983) The isolation and characterization of a mutant strain of *Saccharomyces cerevisiae* that requires a long chain base for growth and for the synthesis of phosphosphingolipids. J. Biol. Chem. **258**, 10200–10203
12 Dickson, R. C. (1998) Sphingolipid functions in *Saccharomyces cerevisiae*. Annu. Rev. Biochem. **67**, 27–48
13 Miyake, Y., Kozutsumi, Y., Nakamura, S., Fujita, T. and Kawasaki, T. (1995) Serine palmitoyl transferase is the primary target of a sphingosine-like immunosupressant, ISP-1/myriocin. Biochem. Biophys. Res. Commun. **211**, 396–403
14 Dickson, R. C. and Lester, R. L. (1999) Yeast sphingolipids. Biochim. Biophys. Acta **1426**, 347–357
15 Hanson, B. A. and Lester, R. L. (1980) The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*. J. Lipid Res. **21**, 309–315
16 Ames, B. N. and Dubin, D. T. (1960) The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**, 769–775
17 van Veldhoven, P. P. and Bell, R. M. (1988) Effect of harvesting methods, growth conditions and growth phase on diacylglycerol levels in cultured human adherent cells. Biochim. Biophys. Acta **959**, 185–196
18 Smith, S. W. and Lester, R. L. (1974) Inositol phosphoryl ceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate. J. Biol. Chem. **249**, 3395–3405
19 Cerbon, J. and Flores, J. (1981) Phospholipid composition and turnover of pathogenic amebas. Comp. Biochem. Physiol. **69B**, 487–492
20 Cerbon, J. and Calderon, V. (1995) Generation, modulation and maintenance of the plasma membrane asymmetric phospholipid composition in yeast cells during growth: their relation to surface potential and membrane protein activity. Biochim. Biophys. Acta **1235**, 100–106
21 Bielawska, A., Perry, D. K. and Hannun, Y. A. (2001) Determination of ceramides and diglycerides by the diacylglyceride kinase assay. Anal. Biochem. **298**, 141–150
22 Merrill, Jr, A. H., Wang, E., Mullins, R. E., Jamison, W. C., Lu Nimbar, S. and Liota, D. C. (1988) Quantitation of free sphingosine in liver by high performance liquid chromatography. Anal. Biochem. **171**, 373–381
23 Merrill, Jr, A. H. (2002) *De novo* sphingolipid biosynthesis: a necessary, but dangerous, pathway. J. Biol. Chem. **277**, 25843–25846
24 Hernandez-Luna, C. E. (1994) Dinámica metabólica de las inositol fosfato ceramidas, Tesis Doctoral, Departamento de Bioquímica CINVESTAV-IPN, México (in Spanish)
25 Shayman, J. A. (1996) Sphingolipids: their role in intracellular signaling and renal growth. J. Am. Soc. Nephrol. **7**, 171–182
26 Wu, W. I., McDonough, V. M., Nickels, J. T., Ko, J., Fischl, A. S., Vales, T. R., Merrill, Jr, A. H. and Carman, G. M. (1995) Regulation of lipid biosynthesis in *Saccharomyces cerevisiae* by fumonisin B1. J. Biol. Chem. **270**, 13171–13178
27 Cerbon, J. and Lopez-Sanchez, R. C. (2003) Diacylglycerol generated during sphingomyelin synthesis is involved in protein kinase C activation and cell proliferation in Madin-Darby canine kidney cells. Biochem. J. **373**, 917–924
28 Sun, Y., Taniguchi, R., Tanque, D., Yamaji, T., Takematsu, H., Mori, K., Fujita, T., Kawasaki, T. and Kozutsumi, Y. (2000) Sli 2 (Ypk1), a homologue of mammalian protein kinase SGKI is a downstream kinase in the sphingolipids-mediated signaling pathway of yeast. Mol. Cell. Biol. **20**, 4411–4419
29 Antonsson, B., Montessuit, S., Friedli, L., Payton, M. A. and Paravicini, G. (1994) Protein kinase C in yeast. Characteristics of the *Saccharomyces cerevisiae* PKC1 gene product. J. Biol. Chem. **269**, 16821–16828
30 Jacoby, J. J., Schmitz, H. P. and Heinisch, J. J. (1997) Mutants affected in the putative diacylglycerol binding site of yeast protein kinase C. FEBS Lett. **417**, 219–222
31 Nomoto, S., Watanabe, Y., Ninomiya-Tsuji, J., Yang, L. X., Kiuchi, K., Hagiwara, M., Hidaka, H., Matsumoto, K. and Irie, K. (1997) Functional analysis of mammalian protein kinase C isozymes in budding yeast and mammalian fibroblasts. Genes Cells **2**, 601–614
32 McMaster, C. R. and Bell, R. M. (1994) Phosphatidylcholine biosynthesis in *Saccharomyces cerevisiae*. Regulatory insight from studies employing null and chimeric sn-1,2 diacylglycerol choline and ethanolamine phosphotransferases. J. Biol. Chem. **269**, 28010–28016
33 Dickson, R. C. and Lester, R. L. (2002) Sphingolipid functions in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1583**, 13–25
34 Hannun, Y. A. and Obeid, L. M. (2002) The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. J. Biol. Chem. **277**, 25847–25850
35 Spiegel, S. and Milstien, S. (2002) Sphingosine 1-phosphate, a key cell signaling molecule. J. Biol. Chem. **277**, 25851–25854
36 Heung, L. J., Luberto, Ch., Plowden, A., Hannun, Y. A. and Del poeta, M. (2004) The sphingolipid pathway regulates Pkc1 through the formation of diacylglycerol in *Cryptococcus neoformans*. J. Biol. Chem. **279**, 21144–21153

Received 23 March 2004/16 November 2004; accepted 24 November 2004
Published as BJ Immediate Publication 24 November 2004, DOI 10.1042/BJ20040475